JOURNAL OF THE
WORLD AQUACULTURE SOCIETY

Vol. 30, No. 2
June, 1999

Application of Polymorphic Microsatellite Loci in a Channel Catfish *Ictalurus punctatus* Breeding Program

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Abstract.—In order to monitor spawning success in earthen ponds, individual channel catfish *Ictalurus punctatus* broodfish were identified prior to stocking by genotype analysis with polymorphic microsatellite DNA markers. Individual spawns were collected and reared in a hatchery, and eight sac-fry per spawn were sampled for genotype analysis. Mendelian inheritance of microsatellite alleles was used to identify parents of each spawn. The probability of detecting a falsely matched offspring and spawning pair was >99%. Average spawning success was 44% (17–80%). Multiple spawning by males was found in seven of the eight ponds sampled. In these ponds, 47% of the males fertilized 2–6 egg masses over 1–8 wk, although most were 2–3 wk apart. Four different spawns were identified as two full-sib families and were probably due to interrupted spawning. Parent/offspring genotype analysis will be useful for production of half-sib families for estimation of genetic components of variation, estimation of effective breeding population, and selection of broodstock for multiple reproductive traits under natural spawning conditions.

Improving reproductive success in farm-raised channel catfish *Ictalurus punctatus* would increase economic efficiency in the U.S. catfish industry. Female spawning success in commercial catfish ponds is estimated at less than 40 to over 80%, depending on water temperatures during the spawning season and condition of broodstock (Tucker and Robinson 1990). Maintenance of non-reproductive broodstock utilizes resources that could be invested in other areas of fingerling production. Selection for reproductive success in a genetic improvement program could increase catfish production efficiency.

The inability to identify spawning or non-spawning broodfish has hindered selection for reproductive success and fecundity in domesticated channel catfish. However, the recent development of molecular markers (microsatellites) in channel catfish (Waldbieser and Bosworth 1997) provides the opportunity for parentage identification. Microsatellite loci consist of short, tandem repeats of genomic DNA that occur throughout vertebrate genomes. The loci contain codominant alleles that can be identified rapidly via polymerase chain reaction (PCR) amplification (Weber and May 1989), permitting the use of non-invasive tissue sampling. Because Mendelian transmission of polymorphic microsatellite alleles provides a high degree of resolution of parent/offspring relationships (Alford et al. 1994; Marklund et al. 1994; Primmer et al. 1995; Heyen et al. 1997; Garcia de Leon et al. 1998), the present research was designed to evaluate the utility of channel catfish microsatellite loci for monitoring reproductive performance under natural pond spawning conditions.

Materials and Methods

Stocking of ponds, spawning, and sampling of spawns took place in the 1996 breeding season (mid-May to mid-July). Two to 3 mo prior to spawning, broodfish (3–5 yr old, 1.2–5.0 kg body weight) were weighed, bled, and stocked in 0.04-ha earthen ponds at an average density of 1,657 kg/ha (range = 847–2,364 kg/ha). The USDA103 strain males and females were stocked in three replicate ponds (A4, A5, and A6), USDA103 males and USDA101 strain females in one pond (C5), USDA101 males and USDA103 females in one pond (D6), and Kansas strain males and females in three replicate ponds (250, 251, and 252) at a sex ratio of 0.9 to 2 females per male. One mL of peripheral blood col-

lected from each broodfish was mixed with 50 μL of 0.34-M dipotassium EDTA and stored at −80 C in 1.5-mL polypropylene tubes. In mid-May, four spawning containers were placed in each pond (100 containers/ha) at an average density of one container/18 kg of fish. The spawning containers were examined two to three times per week for 9 wk and 83 egg masses collected from these ponds were transferred to 75-L tanks in the hatchery. Approximately 50–100 fry were sampled 5–10 d after hatching (before swim-up stage) and stored in 95% ethanol at −20 C.

The blood and fry samples were processed for PCR-based microsatellite genotype analysis. Isolation of genomic DNA from whole blood and individual fry and PCR amplification of microsatellite loci were performed as previously described (Weber and May 1989, Waldbieser and Bosworth 1997). The PCR reactions (15 μL) contained 100-ng genomic DNA, 100-μM Tris [pH 8.3], 50-mM KCl, 1-mM $MgCl_2$, 67-μM dNTP's, 0.4-μM each primer, and 0.5-units Taq polymerase (Boehringer-Mannheim, Indianapolis, Indiana, USA) in a 0.2-mL tube. When possible, two or three loci were amplified in a multiplex polymerase chain reaction. The multiplexed reactions contained the same concentration of all reagents in single primer set reactions, except for additional primer sets. Multiplexes consisted of IpCG0005 (0.40 μM), IpCG0015 (0.33 μM), and IpCG0017 (0.33 μM); IpCG0002 (0.47 μM), IpCG0007 (0.27 μM), and IpCG0009 (0.33 μM); IpCG0001 (0.40 μM) and IpCG0004 (0.33 μM); or IpCG0031 (0.40 μM) and IpCG0043 (0.27 μM). The PCR products were separated by electrophoresis on an ALFexpress DNA Analysis System (Pharmacia Biotech Inc., Piscataway, New Jersey, USA). Reactions for IpCG0008 and IpCG0018 were performed separately but loaded together on the ALFexpress gels.

Parentage identification was performed by comparison of parent and offspring microsatellite genotypes. Microsatellite alleles were identified with AlleleLinks ver 1.00 software (Pharmacia Biotech, Inc.) and exported to a spreadsheet (Microsoft Excel 95). For each locus, unique offspring genotypes were used to construct putative parent genotypes under the assumption of Mendelian inheritance of microsatellite alleles. Comparison of broodfish genotypes with the deduced putative parental genotypes provided identification of parents for each spawn. Parentage exclusion probabilities were calculated for each locus using parental allele frequencies (Gundel and Reetz 1981; Jamieson and Taylor 1997).

TABLE 1. *Microsatellite loci used for identification of spawn parentage.*[a]

Locus	Number of alleles	Heterozygosity	Allele size range (bp)
IpCG0001	15	0.813	206–254
IpCG0002	10	0.716	214–244
IpCG0003	8	0.804	181–209
IpCG0004	15	0.665	132–198
IpCG0005	7	0.326	105–133
IpCG0007	12	0.726	299–343
IpCG0008	15	0.853	122–190
IpCG0009	7	0.516	125–149
IpCG0015	9	0.719	243–279
IpCG0017	9	0.641	163–203
IpCG0018	17	0.879	236–293
IpCG0031	33	0.914	191–329
IpCG0043	18	0.856	110–152

[a] Determined from genotypes in feral, commercial, and research populations.

Results

High levels of microsatellite allele polymorphism in channel catfish (Table 1), and genotyping with several microsatellite loci, permitted the identification of individual broodfish. Parental genotypes were deduced from the Mendelian inheritance of microsatellite alleles from offspring, and the deduced genotypes were compared with broodfish genotypes in each pond (demonstrated in Table 2). Fry mortality prevented sampling from 5 egg masses (A5-5, A5-8, D6-11, 252-7, and 252-8). One spawn (A5-8) was not detected until fingerlings were observed in the pond, but none were ge-

TABLE 2. *Genotypes of offspring in spawn 96-D6-1 and sample of parent genotypes from pond D6.*

96-D6-1	IpCG0001 209/221 (4) 221/221 (4)	IpCG0004 132/132 (5) 132/140 (3)	IpCG0008 146/158 (1) 146/162 (4) 158/182 (1) 162/182 (2)	IpCG0031 199/229 (4) 199/245 (4)	IpCG0043 125/149 (5) 131/149 (3)
196 (M)	221/221	132/140	146/146	195/243	125/125
197 (M)	221/230	132/132	150/162	195/223	134/152
198 (M)	215/230	144/148	130/130	191/231	128/128
199 (M)	209/215	132/144	130/154	231/285	128/134
[a]200 (M)	209/221	132/132	146/182	229/245	125/131
201 (M)	215/245	132/144	166/182	195/195	146/146
202 (M)	221/233	132/132	150/150	259/289	125/125
355 (F)	215/218	132/198	142/154	223/285	122/125
356 (F)	215/221	132/198	134/158	199/285	128/149
357 (F)	215/221	132/132	134/134	199/285	125/149
358 (F)	221/233	140/198	158/158	199/199	137/149
[a]359 (F)	221/221	132/140	158/162	199/199	149/149
360 (F)	215/218	132/198	134/154	223/285	122/125
361 (F)	215/221	132/140	142/158	199/199	137/149

[a] Deduced parent.

notyped. The USDA103 × USDA103 broodfish and spawns in ponds A4, A5, and A6 were initially genotyped with loci IpCG0002, -05, -07, -09, -15, and -17 (Table 1, 3). Unresolved parentage in some spawns from these three ponds required further analysis with IpCG0001, -03, -04, and -08. Both sets of loci were used to genotype the USDA103 × USDA101 matings from pond C5, but IpCG0003 was replaced with IpCG0018 for multiplexing. Both sets of loci identified the same parents for each pond C5 spawn. Therefore the Kansas matings in ponds 250, 251, and 252 were genotyped with IpCG0001, -04, -08, and -18 only. These four loci were also used to analyze the USDA101 × USDA103 matings in pond D6, although some spawns required

TABLE 3. *Parentage exclusion probabilities (P_E) for each pond: Probability of detecting a falsely matched offspring and spawning pair.*

	Locus (IpCG0-)						
Pond	005	015	017	002	007	009	Combined P_E
A4	0.148	0.341	0.584	0.423	0.548	0.322	0.9587
A5	0.309	0.405	0.637	0.497	0.459	—	0.9594
A6	0.166	0.474	0.573	0.607	0.472	—	0.9611
C5	0.496	0.714	0.657	0.765	0.788	0.688	0.9992

	Locus (IpCG0-)							
Pond	001	003	004	008	018	031	043	Combined P_E
A4	0.751	0.679	0.485	0.773	—	—	—	0.9907
A5	0.810	0.727	0.508	0.617	—	—	—	0.9902
A6	0.762	0.614	0.322	0.805	—	—	—	0.9878
C5	0.907	—	0.674	0.906	0.915	—	—	0.9997
D6	0.688	—	0.642	0.842	—	0.866	0.821	0.9996
250	0.856	—	0.706	0.855	0.793	—	—	0.9987
251	0.855	—	0.669	0.868	0.779	—	—	0.9986
252	0.845	—	0.593	0.890	0.838	—	—	0.9989

TABLE 4. *Female and male spawning success in earthen ponds.*

Pond	Stocking rate female/male	Collected	Spawns genotyped[a]	Unique	Sires	Success (%) female/male
A4	16/8 (2.0:1)	5	5	5	5	31/63 (0.5:1)
A5	12/14 (0.9:1)	8	5	5	4	67/[b]
A6	15/12 (1.2:1)	9	9	9	5	60/42 (1.8:1)
C5	30/15 (2.0:1)	26	26	24	8	80/53 (1.5:1)
D6	26/15 (1.7:1)	12	11	11	4	46/[b]
250	30/20 (1.5:1)	5	5	5	3	17/15 (1.1:1)
251	30/20 (1.5:1)	10	10	10	5	33/25 (1.3:1)
252	30/20 (1.5:1)	6	6	6	4	20/20 (1.0:1)

[a] Some spawns not genotyped due to embryo/fry mortality.
[b] Not determined due to fry mortality.

further genotyping with IpCG0031 and -043 to resolve parentage.

Genotyping with several microsatellite loci demonstrated high probabilities of excluding false parentage (Table 3). The probability of detecting a falsely matched offspring and spawning pair in USDA103 × USDA103 matings (ponds A4–A6) was 96% using the first set of 6 loci. However, the second set of loci (IpCG0001, -03, -04, and -08) was more polymorphic in this strain and had a higher probability (99%) of detecting a parent/offspring mismatch. The latter set, including locus IpCG0018 in the place of IpCG0003 to allow multiplexing, was therefore used to analyze the Kansas strain matings (ponds 250–252), and provided a high exclusion probability (99.9%). The USDA103 × Norris strain matings (ponds C5 and D6) also had a high exclusion probability (99.9%) with the same loci as the Kansas strain analysis, or when the IpCG0031/-043 multiplex was tested.

Female spawning success, measured by number of spawns per females stocked, ranged from 17% in pond 250 to 80% in pond C5 (Table 4). Average spawning success was 44%. In pond C5, spawns 5 and 6 were typed to identical parents, as were spawns 12 and 15. The average number of eggs/kg body weight (5,733 ± 1,702) from these four spawns was roughly half the pond average (10,753 ± 486). Fecundity was based on female weights measured at time of stocking.

In seven of the eight ponds, broodfish genotyping revealed multiple spawning by males (Fig. 1), and 18 of the 38 males produced more than one spawn. There was no significant effect of body weight on spawning success by males (data not shown). Eight males fertilized two spawns each, four fertilized three spawns each, four fertilized four spawns each, and two produced five and six spawns, respectively. Multiple spawns from a male were produced from 1–8 wk apart, with the majority 2–3 wk apart. A particular example of spawning dominance was male 200 who fertilized half the spawns in pond D6 over 8 wk (Fig. 1).

Discussion

Microsatellite loci were used to genotype and identify individual channel catfish broodfish stocked in communal ponds. The PCR-based genotype assay provided rapid, repeatable, and unequivocal identification of broodfish spawning in earthen ponds. Different loci were used in each group of fish, mainly due to the uncertainty of the utility of each locus since these analyses were concurrent with the identification of new microsatellite loci. The loci used in the current research were selected according to the level of allelic polymorphism and success in multiplex DNA amplification reac-

		May			June				July										
Pond	Male	20	22	24	28	31	3	6	10	13	17	20	24	27	1	3	5	8	11	15
A4	4	1																		
	2	2																		
	1	3																		
	14/38	4																		
A5	50	1		4																
	91	2																		
	95	3																		
	8								6											
	53															7				
A6	104	1			5															
	27	2																		
	101	3																		
	54		4				7	8							9					
	88						6													
C5	404	1			9	11	13													
	413	2			8		16													
	414	3	7		10	12	15						22							
	406	4,5	6				14													
	407									17			20							
	402										18									
	405											19	21		23		25			
	403														24		26			
D6	200	1	3		7					8	9							12		
	209	2		5																
	204		4		6															
	197														10					
250	1			1																
	37							2						3					5	
	39																	4		
251	84		1	2	3	4														
	56								5											
	82									6	8			9						
	66										7									
	54																		10	
252	103							1				3								
	123									2					4					
	105																		5	
	125																			6

FIGURE 1. *Multiple fertilization of spawns in communal ponds stocked with USDA103 strain (ponds A4, A5, and A6), USDA103 × USDA101 strain (ponds C5, D6) or Kansas strain (ponds 250, 251, and 252) channel catfish. Spawns (shaded box) were identified sequentially within each pond (number within box; fry from spawns A5-5 and D6-11 did not hatch). Male identification number is unique within pond (fish 14 and 38 in pond A4 were both recorded as females at time of blood sampling). For example, in pond A5 male 50 sired spawns collected on 20 May and 24 May, respectively. Boxes connected by outline denote full-sib spawns separated due to interrupted spawning (C5-5 and C5-6; C5-12 and C5-15).*

tions. Spawn parentage could generally be determined with four to six loci when all parents were genotyped, and ponds with higher levels of allelic polymorphism between parents required fewer loci. However, the power of microsatellite genotyping lies in the ability to genotype parents and offspring with as many loci as required to identify individuals. Used in combination, these markers provided high levels of confidence in parentage determination. The parentage exclusion calculations, using

standard formulas based on matching one offspring with parents (Gundel and Reetz 1981; Jamieson and Taylor 1997), were conservative estimates in the present research because information for parent identification was available from several offspring per family, and Mendelian inheritance could be used to reconstruct putative parental genotypes.

Microsatellite analysis confirmed multiple spawning by male channel catfish in one spawning season. This supports previous research (Bondari 1983) that demonstrated no significant reduction in spawning success when males and females were stocked in ponds at densities from 1:1 to 1:4. Multiple spawning by male catfish may allow producers to stock fewer males in production ponds without reducing the number of spawns. Four spawns that were collected from pond C5 as separate egg masses, and initially maintained as separate families, were shown by genotype analysis to belong to two full-sib families. Partial spawning may have been caused by environmental factors or interruption of spawning when the containers were checked. Parents were not identified for several spawns in the other ponds due to fry mortality before sampling. Also, the parents identified for spawn A4-4 were both classified as female. This was probably caused by an error in sex determination at stocking, but broodfish mortality prevented reconciliation of this anomaly. In the current research, ~44% of the females spawned, compared with ~36% of the males.

Half-sib families produced as a result of natural, random spawning reduces the effective size of this breeding population. This may be an important consideration in an applied breeding program when calculating the effective breeding population (N_e) and inbreeding coefficients. Utilizing molecular markers to identify parents provides an accurate method to estimate N_e in random mating populations. These families, however, will provide large numbers of individuals for the estimation of heritabilities from parent/offspring regression. Previous attempts to produce half-sib families useful for estimating components of genetic variation have utilized artificial spawning of individuals and cross-fertilization of gametes. Artificial spawning of catfish often yields poor egg quality and insufficient fry for genetic analyses (Wolters 1993). Parentage verification based on microsatellite marker genotypes provides half-sib families for quantitative genetics experiments from spawns produced under conditions that mimic commercial production, and allows researchers to measure genetic variation in reproductive parameters. Microsatellite-based analysis also allows selection of individual broodstock that have demonstrated successful natural spawning, and avoids possible selection of broodstock that might not have spawned without hormonal stimulation.

Molecular genetic markers, such as microsatellite loci, will be useful for selection of broodstock for multiple reproductive traits. Identification of parents and genotype analysis of spawns will allow measurement of individual spawning success, individual fecundity, and repeated spawning. Measured physiological parameters can also be correlated with annual spawning success for use in a selection index. Due to the costs and expertise required in genotyping, and the large number of broodfish in commercial ponds, this technique is currently more suited for research applications rather than commercial production. Parent/offspring genotype analysis also provides the ability to identify multi-generational families for use in genetic linkage mapping. This will allow researchers to identify genes with major influence on reproductive success, and could lead to marker assisted selection for improved reproductive performance in domesticated catfish.

Acknowledgments

The authors wish to thank Dr. Brian Bosworth for assistance in the selection and stocking of broodfish, and Marjorie Jen-

nings, Terri Griffin, and B.J. Duncan for assistance in sample preparation and microsatellite genotyping. We also thank Drs. Ed Robinson and Craig Tucker for critical review of the manuscript. The primers for the published microsatellite loci are available as Catfish MAPPAIRS™ from Research Genetics, Inc., Huntsville, Alabama, USA. Mention of a trade name, proprietary product, or specific equipment does not constitute a guarantee or warranty by the U.S. Department of Agriculture and does not imply approval to the exclusion of other products that may be suitable.

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